“Error-proofing” and “Future-proofing”: Updating the Bacterial Endotoxins Test with Automation and Recombinant Reagents - Part Deux

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Objective

Use of a recombinant Factor C reagent for endotoxin detection that shows equivalent performance to the Limulus Amebocyte Lysate (LAL) bacterial endotoxin test (BET) helps meet sustainability goals and comply with principles of the 3 Rs (replacement, reduction, refinement of animal use). Application of a robotic platform allows more accurate and precise assay performance, maximizing efficiencies in workstreams. Combining both of these components error-proofs and future-proofs assay execution in your laboratory. This presentation will show data comparing kinetic chromogenic, kinetic turbidimetric, and rFC assays, and discuss both of these concepts to demonstrate laboratory process optimization.

Abstract

The use of robotics and automation reduces error levels common to many manual processes, thus saving time and resources spent on such tasks. Studies comparing manual and automated pipetting and mixing in the BET test show improved precision and accuracy with the automated method. With such automation, laboratory analysts are freed to perform other tasks while a robot performs the manual tasks, resulting in more efficient use of that analyst’s time. Automation of BET methods minimizes the level of interaction with lab analysts, resulting in a reduced level of repetitive motion injuries in line with promulgated regulations for workers protection. In addition to the use of robotics for error-proofing the assay, there is also a need to future-proof the supply chain of reagents. Using a recombinant reagent allows removal of the live animal source thus complying with the “3 R” principles, multiple facility production, and results in a more stable supply chain of a reagent with less lot-to-lot variability. Recombinant Factor C (rFC) based endotoxin detection assays demonstrate equivalent performance when compared to classical LAL-based assays. This presentation will demonstrate that combining the concepts of automation and recombinant reagents results in an error-proofed and future-proofed BET assay process that optimizes laboratory operations.

Recombinant Factor C (rFC) based endotoxin detection assays demonstrate equivalent performance when compared to classical LAL-based assays

The majority of parenteral drugs and implantable medical devices are tested for gram-negative bacterial endotoxin using reagents prepared from the circulating amebocytes found in the blood of the American horseshoe crab, Limulus polyphemus. Variations of this method are described in the USP General Chapter <85> Bacterial Endotoxins Test (1). This chapter describes the gel clot LAL method and the various kinetic and endpoint photometric methods. While the gel clot method is still reserved as the official referee test in cases of dispute, it is implied, by their inclusion, that the photometric methods are acceptable for routine use, if appropriately validated. rFC methods start with the same Factor C as the compendial assays, but do not need the signal amplification steps (**Figure 1**). The assay also yields comparable results as the compendial assay (**Figure 2**). rFC methods offer several distinct advantages over LAL-based methods (2), specifically:

- Eliminates the need to collect and bleed horseshoe crabs, a species facing increased harvesting restrictions
- Provides a non-animal-based method that is needed due to the growing need for endotoxin testing in expanding markets (example: Cell Therapy)
- Protects the supply of endotoxin detection reagents to the pharmaceutical and medical device industries, should a natural or man-made disaster impair the ability of one of the three major LAL manufacturers to harvest horseshoe crabs
- Does not pose a threat to species that rely on the horseshoe crab for survival (i.e. the Red Knot, Calidris canutus)
- Reduces the lot-to-lot variability inherent in animal-derived products like LAL
- Offers more specific endotoxin detection than LAL because it does not contain other horseshoe crab blood components (i.e. Factor G), which can react with non-endotoxin substances (LAL Reactive Materials (LRM)/glucans) and cause false-positive reactions (3, 4). The release of entire lots of parenteral drugs or medical devices may be delayed while the false-positive results are evaluated; in some cases material may be unnecessarily destroyed, raising implications for shortages)
- Allows for the year-round production of the active ingredient (rFC), rather than the seasonal harvesting of horseshoe crab blood during the warmer months
- Does not require toxic or otherwise carcinogenic chemicals to manufacture
- Does not require lyophilization of the active ingredient, resulting in increased manufacturing efficiencies and ease-of-use to end-users. As the active ingredient can be stored in liquid form, this facilitates its use with an on-line endotoxin detection system for water purification systems, which is in line with the FDA Process Analytical Technology initiative

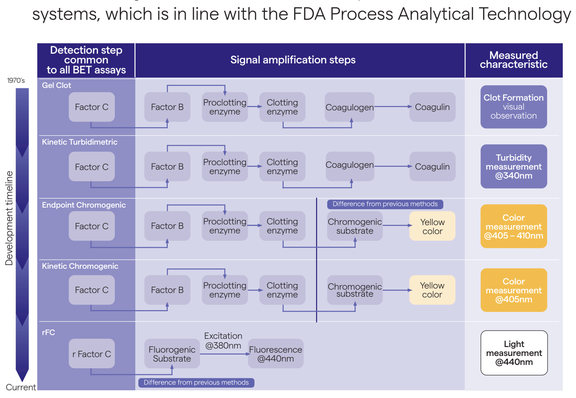

Figure 1. Illustrates that rFC shares the same initial enzyme as traditional LAL assays.

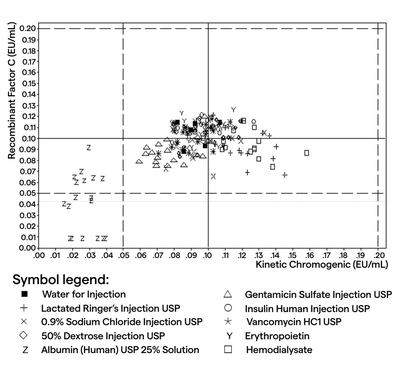

Figure 2. Illustrates the comparability of endotoxin recovery between rFC and classical LAL assays.

Using robotics and automation reduces error levels common to many manual processes, thus saving time and resources spent on retests

Studies comparing manual and automated pipetting, mixing, and standard curve preparation in the BET test show improved precision and accuracy with the automated method.

Our laboratories performed studies to demonstrate that endotoxin standard preparation by automated pipette mixing on the PyroTec® PRO system is equivalent to manual preparation of standards using vortex mixing.

Lonza’s PYROGENT® 5000 Kinetic Turbidimetric LAL Assay Kit provided the necessary endotoxin standard and LAL reagents needed to complete this testing. The CSE standard was reconstituted with the required amount of LAL Reagent Water (LRW) indicated on the Certificate of Analysis for the PYROGENT® 5000 Kit. The CSE vial was vigorously mixed for 15 minutes on a vortex mixer, and a set of standards prepared by dilution with LRW by both the PyroTec® PRO System and manually by a skilled analyst. The PyroTec® PRO System dilutes and mixes by the repeated aspirating and dispensing of 700 µL from a 1 mL total volume seven times for each concentration. Manual dilutions were mixed by vortexing for one minute, as described in the current PYROGENT® 5000 Kinetic Turbidimetric Kit Insert.

The goal in mixing dilutions was to obtain homogeneous solutions at each concentration. Pipette mixing was successful if the standards produced exhibited similar characteristics to those of the manually mixed standards using a vortex mixer. Each standard curve derived by either manual mixing with a vortex mixer, or pipette mixing on the robot had to meet the system suitability criteria to be included in the final analysis. Two different plate readers, the ELx808™ Absorbance Reader (for manual reads) and the Sunrise Absorbance Reader (for both manual and robotic standard curves) were used to read the plates.

The system suitability criteria that must be met in all Lonza kinetic turbidimetric assays were the following:

- Standard Curve with Correlation Coefficient between -1.000 to -0.980, Slope -0.400 to -0.100, and Y-Intercept between 2.500 to 3.500
- Positive Product Control (PPC) with % Recovery between 50% to 200%, and Endotoxin Prediction of a 0.1 EU/mL PPC between 0.5 EU/mL to 2.0 EU/mL
- Endotoxin Standards with % CV of < 10 %
- Run Temperature maintained between 36 to 38°C

This study was comprised of nine independent comparison tests of CSE standard dilutions with concentrations from 10 EU/mL to 0.01 EU/mL and Blank controls, LRW samples, and LRW samples plus PPC. The PyroTec® PRO System followed commands from a WinKQCL® Software template to instruct the analyst where to place dilution tubes and the pre-prepared 50 EU/mL CSE on the deck. While the instrument made the automated dilutions for the standard curve, the analyst manually diluted, and vortex mixed the standards. The analyst manually loaded standards from each method, blanks, and test samples to the assay plate. The PyroTec® PRO System produced standard was loaded into the first two columns with blank and test sample. The analyst loaded the PPC spike from this standard into the sample PPC wells. The manually produced standard, blank, sample, and sample plus PPC spike were loaded into the third and fourth columns. Separate templates for the automation standard with sample and the manual standard with sample were run as a Merged Plate template in each assay run. Assay reports, generated automatically at the completion of each assay run, were evaluated and the data saved for analysis at the completion of all twelve tests.

Results

All system suitability criteria were met in the analyzed runs. Run temperatures were verified to be within 36 to 38°C for all runs. In all runs the lowest standard reaction time was less than the blank reaction time.
The initial data analysis considered the method overlay of all nine standard curves from each of the two preparation methods. These data show little variability from run to run between manually and robotically prepared standard curves. **See Figure 3 and Table 1.**

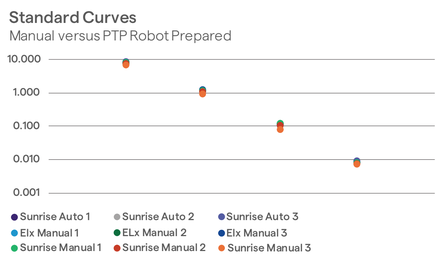

Figure 3. Standard Curve Concentration Comparison.

Nominal Value	Sunrise Auto 1	Sunrise Auto 2	Sunrise Auto 3	Elx Manual 1	ELx Manual 2	Elx Manual 3	Sunrise Manual 1	Sunrise Manual 2	Sunrise Manual 3
10	8.570	9.020	8.960	8.180	8.130	7.850	8.360	8.290	8.770
1	1.180	1.130	1.140	1.240	1.210	1.310	1.180	1.220	1.200
0.1	0.115	0.106	0.107	0.119	0.126	0.120	0.123	0.118	0.103
0.01	0.0086	0.0092	0.0091	0.0083	0.0080	0.0081	0.0082	0.0084	0.0092

Table 1.

Product Testing Using the PYROGENT® 5000 Assay on PyroTec® PRO Robotic System

This study evaluated the automated endotoxin assay as compared to manual endotoxin testing. The goal was to demonstrate that the automation assay produces repeatable results in three different media in-process samples, and one final product, comparing both automated and manual testing. Four different products were tested in triplicate on three different days, using two different plate readers included a panel of 4 samples (**Table 2**). Some samples required preparation prior to testing including pH correction or dilution. The samples included traditionally challenging samples for the kinetic assay due to enhancement or inhibition, samples with known spikes of endotoxin, and were analyzed using PYROGENT® 5000 Kinetic Turbidimetric assay kits.

This study included each of the 4 samples tested at their normal test dilutions, as listed. The analysts tested the entire panel of products including spikes three separate times and on both platforms.

Results

Table 2 shows that results from the manual assay were comparable to those of the automated assay for all products tested. All samples had endotoxin levels below the limit of quantitation at the tested dilution. **Figure 4** shows that the PPCs for the automated method are slightly tighter than the PPCs for the manual process, demonstrating less variability of results in automated assays than manual assays.

Sample name	Manual or Auto	Reader	Dilution	EU/mL	Mean PPC	PPC % Range Low	PPC % Range High
CHE2 basal w/o Heparin a	Auto	Sunrise	100	<1.00	0.131	124%	135%
CHE2 basal w/o Heparin a	Manual	Elx	100	<1.00	0.13	127%	133%
CHE2 basal w/o Heparin a	Manual	Sunrise	100	<1.00	0.102	95%	115%
CHE2 basal w/o Heparin b	Auto	Sunrise	100	<1.00	0.138	127%	144%
CHE2 basal w/o Heparin b	Manual	Elx	100	<1.00	0.143	133%	156%
CHE2 basal w/o Heparin b	Manual	Sunrise	100	<1.00	0.119	114%	127%
CHE2 basal w/o Heparin c	Auto	Sunrise	100	<1.00	0.137	132%	139%
CHE2 basal w/o Heparin c	Manual	Elx	100	<1.00	0.15	146%	153%
CHE2 basal w/o Heparin c	Manual	Sunrise	100	<1.00	0.131	119%	144%
IMDM a	Auto	Sunrise	10	<0.1	0.152	142%	157%
IMDM a	Manual	Elx	10	<0.1	0.127	119%	140%
IMDM a	Manual	Sunrise	10	<0.1	0.114	109%	121%
IMDM b	Auto	Sunrise	10	<0.1	0.161	154%	164%
IMDM b	Manual	Elx	10	<0.1	0.13	120%	143%
IMDM b	Manual	Sunrise	10	<0.1	0.131	118%	148%
IMDM c	Auto	Sunrise	10	<0.1	0.145	141%	147%
IMDM c	Manual	Elx	10	<0.1	0.137	121%	148%
IMDM c	Manual	Sunrise	10	<0.1	0.112	104%	125%
NaCl 0.9 % a	Auto	Sunrise	100	<1.00	0.113	105%	117%
NaCl 0.9 % a	Manual	Elx	100	<1.00	0.123	117%	127%
NaCl 0.9 % a	Manual	Sunrise	100	<1.00	0.115	109%	122%
NaCl 0.9 % b	Auto	Sunrise	100	<1.00	0.114	108%	117%
NaCl 0.9 % b	Manual	Elx	100	<1.00	0.129	116%	137%
NaCl 0.9 % b	Manual	Sunrise	100	<1.00	0.114	110%	118%
NaCl 0.9 % c	Auto	Sunrise	100	<1.00	0.111	109%	112%
NaCl 0.9 % c	Manual	Elx	100	<1.00	0.108	105%	110%
NaCl 0.9 % c	Manual	Sunrise	100	<1.00	0.094	91%	98%
Trypsin-Versene a	Auto	Sunrise	1000	<10	0.12	115%	122%
Trypsin-Versene a	Manual	Elx	1000	<10	0.149	145%	152%
Trypsin-Versene a	Manual	Sunrise	1000	<10	0.105	99%	110%
Trypsin-Versene b	Auto	Sunrise	1000	<10	0.121	117%	123%
Trypsin-Versene b	Manual	Elx	1000	<10	0.132	128%	134%
Trypsin-Versene b	Manual	Sunrise	1000	<10	0.109	102%	121%
Trypsin-Versene c	Auto	Sunrise	1000	<10	0.131	117%	138%
Trypsin-Versene c	Manual	Elx	1000	<10	0.152	145%	163%
Trypsin-Versene c	Manual	Sunrise	1000	<10	0.121	119%	123%

Table 2.

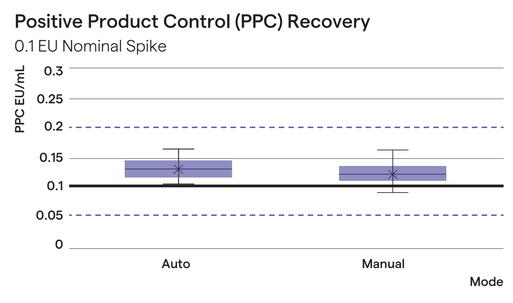

Figure 4.

Conclusion

Overall, the standard and sample results were very similar between Automation and Manual methods of performing the PYROGENT® 5000 Kinetic Turbidimetric Assays.
All samples had passing results at the routine test dilution in each method and were below the Level of Quantitation (LOQ) for the dilution.
The PPC % recovery in the samples was slightly above 100 % in both the manual method and the automated method. The PPC % CV were low for both methods with automation producing tighter results overall.
All of the standards met the specifications for passing in the PYROGENT® 5000 Kinetic Turbidimetric Assay. The standards were very similar between the methods. The automation standard’s Y-intercept values were slightly broader, but the % CV values among replicates were tight.

Putting it All Together: Error-proofing and Future-proofing

The laboratory performed a reliability study using KINETIC-QCL® Kinetic Chromogenic Assay, PyroGene® Recombinant Factor C (rFC), and PYROGENT® 5000 Kinetic Turbidimetric on the PyroTec® PRO Robotic Solution. Once programmed, the robot was able to repeatedly and accurately perform dilution series by pipetting Control Standard Endotoxin (CSE) in a series of 1:10 dilutions, then plate those dilutions into the proper wells, pipette the endotoxin detection reagents into each well, and then move the prepared plate into the either the absorbance reader (for KINETIC-QCL® or PYROGENT® 5000 Assays), or the fluorescence reader (for the PyroGene® Assay). This saved approximately one hour of analyst time over manual preparation in each assay setup. Once those steps were completed, the WinKQCL® Software took over to monitor the readers temperature, timing, wavelength, and absorbance or generated fluorescence to calculate the amount of endotoxin in each well. As shown, the box and whisker graphs **Figures 5, 6, and 7** illustrate the precision and accuracy that may be achieved using a combination of the animal-friendly and renewable PyroGene® Assay and the PyroTec® PRO Robotic System.

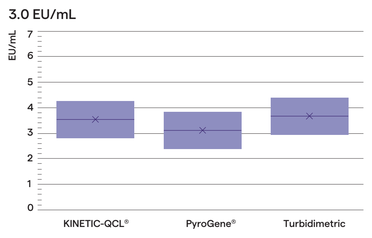

Figure 5.

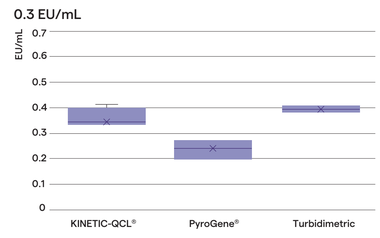

Figure 6.

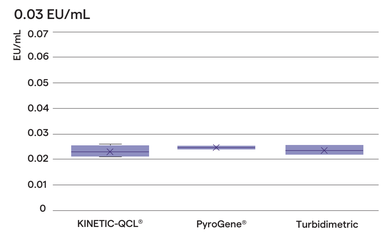

Figure 7.

As demonstrated in this presentation, removing the analyst from such labor-intensive tasks and relying on robotics reduces the chance for human error in the process, and produces less assay variation thus “error-proofing” the assay. Relying on recombinant sources of materials used in endotoxin testing provides a renewable endotoxin detection assay guarding the safety of parenteral products without relying on animals as a source of raw materials, thus “future-proofing” the availability of the reagents. These combined attributes make the use of the PyroGene® rFC Assay with the PyroTec® PRO Robotic Solution ideal for high-throughput laboratories.

References

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